## Ligation Based Cloning

Digestion (restriction enzymes):

Purified DNA fragments are digested with the appropriate restriction enzymes prior to ligation. The reaction mixture for restriction digest is prepared as followed in Table 7. Here a double digest is performed, so two restriction enzymes working at the same time. Use enzymes of same company and check in advance buffers for restriction and star activity.

**Table 7: composition of restriction digest reaction**

| Component | Concentration in reaction mixture | Volume in 20 µL reaction mixture |
|---|---|---|
| **Nuclease free Water** | | x |
| **Fast Digest Buffer** | | 2 |
| **PCR-Product** | | 500 – 1000 ng |
| **Fast Digest Reaction Enzme 1** | | 1.5 |
| **Fast Digest Reaction Enzme 2** | | 1.5 |

Restriction digest is performed for 1 – 2 h with the purified vector and the purified insert in separate reactions. Afterwards the enzymes are denatured at 80°C for 20 min or immediately purified. Digested DNA is agarose gel purified with the Gel Extraction Kit.

Purifications (Kit, Gelex):

Ligation:

For ligation T4 DNA ligase is used to ligate 50 ng of the amplified and purified plasmid vector backbone with the insert DNA in threefold or fivefold molar excess (see Table 8). The determination of insert-DNA volume is carried out by the following equation:

$$V_{insert} = 1/c_{insert} * x * 50\ ng * s_{insert}/s_{vector}$$

x = factor of insert molar excess
v = volume
s = size [bp]

**Table 8: ligation reactions with different ratios of vector:insert**

| Component | 1:3 | 1:5 | 1:0 (control) |
|---|---|---|---|
| **Nuclease free Water** | x for total 20 µL | x for total 20 µL | x for total 20 µL |
| **Vector backbone** | 50 ng | 50 ng | 50 ng |
| **Insert** | x µL | x µL | - |
| **T4 Ligation Buffer** | 2 µL | 2 µL | 2 µL |
| **T4 DNA ligase** | 1 µL | 1 µL | 1 µL |

The reaction is carried out for 20 min for 1 h, at roomtemperature (RT) or overnight at 16°C. Subsequently, 5 µL of the reaction mix is used to transform electro-competent E.coli BL21 Gold cells.

Ligation within a PCR product carrying both restriction sites is carried out in the following reaction mixture shown in Table 9.

**Table 9: reaction mixture for ligation within a PCR product**

| Component | V / µL |
|---|---|
| **Nuclease free Water** | 40.0 |
| **T4 Ligation Buffer** | 5.0 |

| | |
|---|---|
| **Digested DNA** | 4.0 (~56 ng) |
| **T4 DNA ligase** | 1.0 |